

The University of Chicago

CHARACTERISTICS OF SMALL COLONY
VARIANTS WITH SPECIAL REFERENCE
TO SHIGELLA PARADYSENTERIAE
SONNE

A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE BIO-
LOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF HYGIENE AND BACTERIOLOGY

1935

By
BEN D. CHINN

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CHARACTERISTICS OF SMALL COLONY VARIANTS WITH SPECIAL REFERENCE TO SHIGELLA PARADYSENTERIAE SONNE

BEN D. CHINN

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To the common classification of colony types, smooth (S), rough (R) and their intermediate stages, there has been added another type originating from the work of Hadley, Delves and Klimek¹ with *S. dysenteriae* Shiga. These workers have called this the G type because of its hypothetical relationship to the gonidial stage. They regard the G type as a culture state intermediate between a filterable virus stage and the "normal" and that "the G forms are normal products of dissociative activity in the mother culture, and represent, in the bacterial development, a definite cyclostage which seems to show some analogies to the gonidial forms of the higher fungi." While there may be some doubt as to whether the G form is really a stage in the life cycle of development there can be no doubt as to its occurrence.

A few earlier reports of apparently similar forms preceded Hadley's work but since then G forms have been described for a number of species of bacteria. Edwards² described "dwarf" colony variants of *Shigella equirulis* in a study of a disease of foals. Koser and Dienst³ obtained several G colony variants of *S. paradyseae* Sonne in aging casein digest broth from old agar slant cultures and from agar plates streaked from stock cultures. Hoffstadt and Youmans⁴ isolated filterable G dissociants of *Staphylococcus aureus*. A G form of *E. typhosa* was obtained by Hadley and Carapetian⁵ which was morphologically identical with the normal strain, filterable through an N filter but could not revert to the original culture type. Small colonies of *Bacillus megatherium* were obtained by Rettger and Gillespie⁶ when serial transfers were made in lithium chloride broth. Roos, Reichel and Clark⁷ found G variants of hemolytic streptococci which retained their agglutinative and biochemical properties and lost their virulence but not their toxigenicity. The G strain encountered by Roe⁸ in the dissociation of *Cl. welchii* was culturally and serologically identical to the S and R forms but avirulent for guinea pigs. The G variants isolated by Kopeloff⁹ of *L. acidophilus* were

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1. J. Infect. Dis. **48**: 1, 1931.
2. Kentucky Agric. Exper. Sta. Bull. 320, 1931.
3. J. Infect. Dis. **54**: 131, 1934.
4. J. Infect. Dis. **51**: 216, 1932.
5. J. Bact. **25**: 94, 1933.
6. J. Bact. **26**: 289, 1933.
7. J. Bact. **27**: 99, 1934.
8. J. Bact. **27**: 46, 1934.
9. J. Infect. Dis. **55**: 368, 1934.

found to be non-filterable. Swingle¹⁰ isolated a number of G strains from *Staph. aureus* cultures which reverted to the normal quite readily and were antigenically similar to the strains from which they were derived. They could not be recovered from Berkefeld filtrates.

In this investigation G colony forms of *Shigella paradysenteriae* Sonne were studied with regard to their occurrence in old agar slant cultures and a comparison was made with the normal with respect to physiological and antigenic properties and filterability. A determination of the electrophoretic velocities of the cells comprising the different colony forms was also made.

THE ISOLATION OF G COLONIES

Ten strains of *Shigella paradysenteriae* Sonne from the collection studied by Koser and his coworkers³ were selected for this investigation. Preliminary experiments had indicated that G colony forms might be obtained by streaking agar plates with small amounts of growth from old agar slant cultures. The ten strains were cultured on nutrient agar slants for 24 hours at 37 C. and then aged at room temperature for 2 to 5 months. From these cultures transfers were made by streaking upon nutrient agar plates. The plates were incubated at 37 C. for five days. They were examined at 24 hour intervals for small colonies of the G type with the aid of a colony microscope with 30X magnification. Since it was desired to obtain some idea of the relative proportion of G colonies to those of the normal size, counts were made of all colonies present. Any minute colonies which were found were selected for streaking to agar plates for isolation and further study. A colony was considered of the G type if its size did not exceed 200 microns and if the subculture remained minute after 48 hours incubation.

A total of 32,000 well isolated colonies was counted among which were 621 small colonies. Not all of these could be considered true G colonies however. Of the 621 small colonies 76 were transferred for further study and only 35 of them continued to grow in the typical G form. The others reverted to normal size S colonies on primary transfer. Many of the small colonies obtained on the primary plate from an old culture possess the ability to regain their normal colony development very readily. Other cells continue to form minute colonies even after numerous transfers and long incubation periods. These were considered to be the G type colonies. The proportion of G colonies in old cultures appears to be about one in one hundred.

The possibility of the G variant being present in young cultures was also investigated. Eighteen to 24 hour cultures were streaked in the usual manner on nutrient agar plates and the plates examined at 24 hour intervals during an incubation period of 5 days. On plates containing a total of 3,425 isolated colonies the G type was never found. Thus, cells possessing the potentiality of developing G colonies were not encountered in 18 to 24 hour cultures, but did appear in about 1% of the cases when old cultures were examined.

Another method was tried for the isolation of G colonies. Hadley¹ made serial transfers at 24 hour intervals in nutrient broth and in lithium chloride

10. J. Bact. 29: 467, 1935.

broth and after the sixth to the tenth transfer often obtained G colonies on agar plates. Using this procedure, three strains of *S. paradysenteriae* Sonne were passed serially at 24 hour intervals through nutrient broth and plates were streaked at the time of transfer from each 24 hour culture. During 25 serial transfers no G colonies were obtained.

Other variant colony forms.—An unusual change took place in the structure of the normal sized S and R colonies transferred from the ten original aged cultures during the regular process of isolation. The plates, streaked in duplicate, were observed after 24 hours and only S and SR colonies were found. After 72 hours the following colony types were present: (1) very rough colonies, (2) colonies showing central lysed areas, (3) colonies with secondary growths or daughter colonies and (4) G type colonies. The various kinds of colonies were picked and streaked on agar plates. Each of these different types produced, upon transfer, only S and SR colonies. The daughter colonies were probably similar to those described by Massini¹¹ in colony changes observed in *B. coli mutabile*, Dienst¹² for *S. paradysenteriae* Sonne and by Hall¹³ for several other organisms. Colonies showing "lysed areas" or the "erosive phenomenon" were observed by Preisz¹⁴ in a study of the anthrax bacillus, by Hadley¹⁵ in *B. pyocyaneus* and by Dienst¹² in *S. paradysenteriae* Sonne.

COLONY AND CELL MORPHOLOGY

Seven G strains were selected from those isolated for further study. These variants, kept on agar slants as stock cultures, retained their small colony form throughout the work. To this group were added two other strains obtained by Koser and Dienst,³ so that nine in all were studied. These were numbered G1 to G9 for further reference.

Normal colonies of *S. paradysenteriae* Sonne were generally from 1.5 to 2.5 mm. in diameter while the small colony variants were often invisible to the unaided eye when grown on nutrient agar at 37 C. for 48 hours. The smallest variant produced colonies from 0.012 to 0.066 mm. but colonies of other variants were occasionally as large as 0.20 mm. On nutrient agar each strain maintained a rather constant colony size within limits characteristics of each strain. The G colonies were generally well isolated, round, smooth, convex and fairly translucent.

Cell morphology was exceedingly variable. While the normal cell is a rod about 3 microns long the variant can be found as long, slender filamentous forms, very short rods, swollen and minute cocci and club-shaped cells. All were gram-negative and showed a granular appearance when stained. In hanging drop preparations this same morphology could be seen.

In microculture the method of cell division in the variant appeared to be different from that of the normal. The microculture was prepared by placing

11. Arch. f. Hyg. **61**: 250, 1907.

12. J. Bact. **26**: 489, 1933.

13. J. Bact. **29**: 13, 1935.

14. Centralbl. f. Bakt. **35**: 280, 1904.

15. J. Infect. Dis. **34**: 260, 1924.

a small loop-full of veal infusion broth on a sterile cover slip and a small amount of a 24 hour culture was suspended in it. To this were added two or three loops of 1% veal infusion agar and the whole emulsified. After hardening, a hanging drop slide, with a vaseline seal around the concavity, was gently pressed over the cover slip and then, with the proper side up, was placed in a warm stage chamber at a temperature of 37 C. After an incubation period of two hours, to allow for the lag phase, a number of cells were observed with the high dry objective (430X).

In the normal strains the cell first lengthens until its size has increased to about one and one-third its original length. At one end a constriction is formed producing a bud-like projection. Into this the protoplasm seems to flow for there is a gradual lengthening of this bud. When it is about one-half the size of the whole cell the constriction narrows and finally a new cell breaks off. The time required for cell division as observed by this method is from 12 to 20 minutes.

In the G variant there was a slight lengthening of the cell, then a constriction appeared at the center which gradually narrowed until the cell divided into two equal parts. The time for cell division was much longer, one and one-half hours to four hours and fifteen minutes.

It is generally believed that the genus *Bacterium* divides by simple fission but under certain conditions and possibly during the maximum growth rate normal cells may divide by a process similar to budding.

GROWTH RATES OF G COLONY VARIANTS

The G colony variants under examination did not grow rapidly in nutrient broth and the addition of various enriching substances often failed to increase growth appreciably. A comparison of the growth rates and minimum generation times of the normal and G forms was made.

The size of the G colonies, as determined by streaking on nutrient agar before inoculating into nutrient broth in which the rates were determined, was as follows: (in mm.)

Strain	Maximum	Minimum	Average
G1	.066	.012	.04
G2	.16	.05	.10
G3	.20	.08	.14
G6	.12	.02	.07
G7	.18	.05	.08

The strains were inoculated from 24 hour broth cultures into flasks of nutrient broth. The counts of viable bacteria were made immediately after inoculation, after 4, 10, 24 and 48 hours and after 3, 4 and 6 days at 37 C. The pour plate method was used in these determinations and the resulting colonies were counted after 24 hours in the case of the normal and after 48 and 96 hours with the G forms. For the detection of the G colonies in the agar plates the aid of the colony microscope was required.

The minimum generation time for the normal strains was found to be about 36 minutes during the logarithmic growth phase. The size of the colonies prior to the determination was 1 mm. to 4 mm. the average being

2.5 mm. The rate of growth of the G strains was found to be much slower and the logarithmic growth phase, usually taking place between 2 and 8 hours in the normal strains, took place between 4 and 24 hours in the G strains.

A reduction in cell count was found in strains G1, G6, and G7 during the first 4 to 10 hours. It is striking to observe that those G strains producing the smallest colonies and which are not easily brought to the normal size show this prior reduction in numbers and a longer logarithmic phase. The colonies of the G strains remained small on plates streaked from these cultures so that the increase in numbers could not be attributed to the sudden appearance of normal cells. The minimum generation times were as follows:

G1	3 hr., 54 min.
G2	1 " 46 "
G3	1 " 54 "
G6	1 " 54 "
G7	2 " 24 "

This is a decided contrast to the 36 minute generation time of the two normal strains.

These growth rates may be significant in explaining the slow metabolism in sugar mediums as well as the possibility of overlooking G forms on plates. In mixtures, the G colony cells would be completely overgrown by the normal during the first 24 hours but since the G forms persist at their maximum numbers for a greater length of time it should be possible to isolate them from old cultures rather than from young ones, assuming that the G would be able to continue development when mixed with more rapidly growing normal cells.

THE REVERSION OF THE G VARIANT TO THE NORMAL FORM

The reversion of the G colony variant to the normal colony size with corresponding biochemical and serological reactions was considered essential for the establishment of the validity of the G variants. It is recognized, of course, that this excludes certain strains which may be true variants but do not revert by any of the methods at our disposal. Under such conditions there is always the uncertainty as to whether one is dealing with a variant or a contaminant.

Strains G2 and G3 were brought back to normal size by prolonging the incubation period in nutrient or veal infusion broth from two days to four or five days and also by 15 to 20 serial transfers on nutrient or veal infusion agar as had been previously accomplished by Koser and Dienst.³ G7 and G9 completely reverted to the normal size several times when plates were incubated for five days. In 48 hours the average colony size was 0.064 mm. while after five days there was an increase to an average of 2.0 mm. Transfer of the enlarged colony to nutrient agar reproduced normal colonies in 48 hours. Fermentation by the reverted G7 and G9 strains was then normal and the maximum agglutination titer was 1-2,560 and 1-1,280 respectively with normal antiserum. G8 reverted very rapidly and the small colony variant was lost.

Strains G1, G4, G5 and G6 retained their small colony size and several additional methods were tried to produce colonies of the usual size. Tryptophane broth, brain infusion broth, sterile filtrates of normal cultures, blood agar and hormone-ascitic fluid broth were used to stimulate increase in colony size but without much success. Greatly enriched mediums did have some stimulating effect upon mass growth but colony size did not increase to normal, though slight increases were obtained with some mediums. Ascites-fluid broth and blood agar were most effective in this respect. These four strains, therefore, while believed to be true variants of *S. paradysenteriae* Sonne must be viewed with some doubt.

FERMENTATION REACTIONS

A diversity of results have been obtained by those who have studied the fermentation reactions of small colony variants. Edwards² found that his dwarf colonies of *Shigella equirulis* did not grow sufficiently in sugar broth to produce any reaction. Some of the G forms of *S. dysenteriae* Shiga obtained by Hadley¹ fermented dextrose and maltose but not mannite while others did not ferment any sugars. Roos, Reichel and Clark⁷ found that the G forms of hemolytic streptococci fermented the same sugars as did the normal strains. Koser and Dienst³ noted that the G strains of *S. paradysenteriae* Sonne for the most part fermented the same sugars as the normal but took a longer time to do so. Swingle¹⁰ obtained similar results for the G variants of *Staph. aureus*. Raven¹⁶ in a study of the dissociants of *N. gonorrhoeae* found that the G variant did not ferment any sugars.

Preliminary work on the fermentation reactions of the G strains showed that their ability to ferment dextrose, lactose and sucrose was greatly retarded as compared to the normal or entirely lost. This may have been due, in part at least, to slow or very scanty growth. On the addition of 10% serum to the medium both growth and fermentation were stimulated.

Of the G strains, whose fermentation reactions have been studied, G2, G3 and G7 could be brought back to the normal colony size by methods described in the preceding section. After reversion their fermentation reactions were determined in order to ascertain whether reversion to normal colony form brought reversion to normal fermentation reactions. The normal sized colony strains obtained from G3 and G7 were inoculated into 20 different sugar broths and at the same time comparative tests with the normal stock strains were made. The same sugars were fermented in both cases although the fermentation of several sugars by the reverted strains was slightly slower.

For the reactions of the small colony variants themselves strains G1, G2, G3, G6 and G7 were inoculated into two sets of sugar broths, one containing 10% serum. Table 1 shows the results of those fermentation tests. It is apparent that with strains G1, G6 and G7 some sugars were fermented in serum medium that were not fermented in the serum free medium. Of the 20 sugars used G7 fermented 14 in the serum broth and 10 in the plain sugar broth all of which were fermented by the normal strains. G6 fermented 9 sugars in

16. J. Infect. Dis. 55: 328, 1934.

the presence of serum and none in its absence. G1 fermented 8 sugars with serum and 5 without serum. A long incubation period was necessary to show the fermentation characteristics of the small colony forms for in almost all cases negative results would have been obtained if incubated for the usual length of time. In some instances the fermentation of the sugars required 20 to 30 days.

TABLE 1.—*Reactions of G Strains in Sugar and Serum-Sugar Broths**

Strain	G1		G6		G7		G3		G2	
	SB	SSB	SB	SSB	SB	SSB	SB	SSB	SB	SSB
Dextrose	a6	A6	0	A6	A4	A10	A3	A4	A1	A1
Lactose	0	0	0	a10	0	A21	A15	A21	A6	A8
Sucrose	a6	A28	0	a10	a28	A21	A28	A21	A8	A8
Dextrin	0	a28	0	A8	0	A28	0	A8	A1	A1
Arabinose	A8	A8	0	a8	A23	A28	A3	A6	A1	A1
Galactose	0	A8	0	A8	A6	A28	A2	A8	A1	A1
Trehalose	0	0	0	0	0	0	A3	A8	A1	A1
Raffinose	0	0	0	0	0	A28	a30	0	A6	A10
Mannose	0	0	0	a28	A2	a21	A2	A8	A1	A1
Mannite	0	0	0	0	A3	A30	A2	A6	A1	A1
Levulose	A6	A20	0	A20	A2	A6	A2	A2	A1	A1
Dulcitol	0	0	0	0	0	0	0	0	0	0
Glycerol	0	0	0	0	0	0	A6	a10	A6	A10
Maltose	a10	a28	0	A6	0	A28	A6	A4	A1	A1
Xylose	0	0	0	0	A28	A30	0	0	a1	a1
Salicin	0	0	0	0	A4	A28	0	0	A17	0
Rhamnose	0	a8	0	0	A8	A32	A2	A2	A1	A1
Inulin	0	0	0	0	0	0	0	0	0	0
Sorbitol	0	0	0	0	0	0	0	0	0	0
Adonitol	0	0	0	0	0	0	0	0	0	0

* Observed for 35 days. Numbers indicate the time in days to produce complete (A = acid) or partial (a) fermentation. Negative results are indicated by ciphers. SB = Sugar broth, SSB = Serum-sugar broth.

Those G strains which could revert to the large colony form, G2, G3 and G7, fermented the sugars rapidly while the strains which consistently produced very small G colonies fermented the sugars slowly and in some cases only partially. The slow fermentation is probably due to the slow growth of the G strains. Inulin, sorbitol, dulcitol, adonitol and xylose which were not fermented by the normal strains were not fermented by the G strains. Salicin, fermented in ordinary sugar broths by G2 and G7 is not normally fermented. The addition of serum stimulates fermentation by those strains which do not act upon sugar in ordinary nutrient sugar broth.

At various times during the incubation period plates were streaked from the fermentation tubes to determine colony size. In all cases no change in colony size had taken place. This shows that fermentation was carried out by the small colony variant and not by the reverted form.

It can be said that those G strains which revert to the normal colony form ferment all or almost all of the sugars normally fermented by the corresponding normal strains. The small colony variants which do not readily change to the normal form grow very slowly and ferment relatively few sugars. In these cases the addition of serum to the sugar broth usually resulted in somewhat better growth and the fermentation of a wider range of sugars.

SEROLOGICAL RELATIONSHIPS

Agglutination tests.—Preliminary agglutination tests of the G colony forms with the antiserum of normal strain 269 had indicated a relationship, in some instances, between the G and normal strains of *S. paradysenteriae* Sonne. G2, G3, G7 and G9 had given definite positive results with low titers

and, as previously noted in the section on reversion, higher titers when reversion to the normal colony form had taken place. The G5 and G6 strains had given doubtful results but these strains did not revert to the normal colony size. The agglutination tests of G1 and G4 were always negative and the fermentation tests were indefinite.

Several of the G strains under consideration G3, G6 and G7 were therefore taken to determine their relationship to the normal strains of *S. paratyphosa* Sonne. G3 reverted to the normal readily and has been shown to be related to it. G7 was brought back to the normal several times but generally persisted in the small colony form. G6 could not be made to grow as a larger colony.

For the immunization of rabbits the cultures were grown on nutrient agar in the case of the normal and G3 strains and on veal infusion agar for G6 and G7. The infusion medium was necessary to obtain sufficient growth. In the variant strains the G colony size of the stock cultures was maintained throughout the immunization period. The average colony size during this time was as follows: (in mm.)

Normal colonies	2.5
G3	0.18
G6	0.06
G7	0.08

Table 2 presents the results of the agglutination reactions between the normal and G antigens and their respective normal and G immune serums. This table shows that the normal immune serum agglutinated three normal colony strains and strain G3 which produced colonies of about 0.18 mm. in diameter. The latter strain could readily revert to the normal sized colony under suitable conditions. This immune serum had little or no effect upon the other two G antigens.

TABLE 2.—*Agglutination Titer of Serums Prepared with Normal and G Antigens**

Serum	Normal (269)	Normal (Matheson)	Normal (St. Andrews)	G3	G7	G6
Normal (269)	5120	2560	2560	5120	10	10
G3	640	1280	640	10240	0	80
G7	80	40	40	40	160	0
G6	80	0	20	40	0	1280

* G3 isolated from 269, G7 from Matheson and G6 from St. Andrews normal strains.

The immune serum of the larger colony variant, G3, agglutinated its homologous antigen to a higher titer than the normal. G7 was not agglutinated and G6 was agglutinated slightly in the lower dilutions.

The immune serum of G7 was tested against its homologous antigen, three normal strains and two other G strains. This serum definitely agglutinated its homologous antigen and the normal strain (Matheson) from which it was derived was partially agglutinated to a titer of 1-40. The other normal strains and G3 were also agglutinated and the minute colony form G6 was not.

The immune serum produced by G6 showed slight agglutination with two normal strains, including the one from which G6 was isolated (St.

Andrews), and G3, but there was no action on G7. The homologous antigen was agglutinated to a titer of 1-1,280.

It is clear from these experiments that strain G3 is definitely related to the normal strains of *S. paradysenteriae* Sonne. G7 has shown relationships to the normal upon reversion but in the small colony state agglutination and fermentation reactions of this strain are considerably reduced. Whether G6 is similarly related to this organism cannot be stated with surety.

Complement fixation tests.—A further study of the relationship between the normal strains and the G variants by complement fixation reactions was made. The immune serums used were the same as those used in the agglutination tests. The antigens were prepared in the following manner. Strains 269 (normal) and G3 were cultivated on nutrient agar, G7 on veal infusion agar and G6 on ascitic fluid veal infusion agar. As noted before, this difference in the medium was necessary to secure enough growth of certain of the G forms. The growth on the plates was harvested by adding sterile distilled water and swabbing off the growth. Equal quantities of packed cells were taken and suspended in 15 cc. of water. All suspensions were steamed by Arnold sterilization for two hours. The cultures were found to be sterile and were preserved in phenol. The autolysed cells were centrifuged and the supernatant liquid was used as the antigen in these experiments.

Table 3 shows the results obtained in cross complement fixation tests using the immune serum against homologous and heterologous normal and G antigens. These experiments were repeated and confirmed.

TABLE 3.—*Complement Fixation Titer of Serums Prepared with Normal and G Antigens*

Serum	Normal (269)	G3	G7	G6
Normal (269)	400	400	40	40
G3	200	200	40	40
G7	10	20	40	40
G6	0	0	40	400

From these results there appears to be a definite interrelationship between the normal and the G strains. With the homologous antigens complement was fixed in higher dilutions than with the heterologous antigens. The normal and the larger colony G variant, G3, showed a close relationship to the other G variants. Strains G6 and G7 showed cross relationships. G7 immune serum fixed complement with the antigens of the normal and G3 strains in low dilutions only but G6 to a higher dilution. G6 fixed complement to a fairly high dilution with G7 but not at all with 269 (normal) and G3. The smaller the colony the less apparent is the relationship to the larger G and normal strains.

It is generally believed that the complement fixation reaction is not as strictly specific as the agglutination reactions and the above complement fixation tests indicate a broad relationship among the strains studied. The specificity as shown by the complement fixation reaction may not be as true as it appears to be. However, if the complement fixation reaction shows more of the group relationships than strict strain specificity the normal and G strains fall in an antigenically related group.

METHYLENE BLUE REDUCTION EXPERIMENTS

The methods of Quastel¹⁷ and Kendall¹⁸ in the study of resting cells have been applied here to determine the activity of normal and G forms of *S. paradysenteriae* Sonne and of *Staphylococcus aureus* in reducing methylene blue. The term "resting" bacteria was first used by Quastel¹⁹ and "designates organisms that are fully mature and endowed with their full potentiality for metabolism but are constrained from multiplication by the withholding of substrates essential for their continued growth."

Resting bacteria will allow a study of the changes which take place in a substrate by the non-growing cell. The conditions essential for this type of work are absence of nutritive nitrogen, anaerobic conditions, short periods of time (usually 30 minutes), relatively high temperatures so that growth will not occur and the use of substrates which will not induce anaerobic growth of the organism under consideration. In this way bacteria act like enzymes or catalysts (Quastel).¹⁷

It is well known that certain substances alone are not capable of reducing methylene blue. Washed muscle tissue does not reduce methylene blue easily but if sodium succinate is present the dye is quickly reduced. Similarly a suspension of resting cells of *S. paradysenteriae* Sonne in methylene blue will not reduce the dye but if some hydrogen donor such as glucose is present the methylene blue is readily reduced demonstrating the enzymatic action of the cells in which hydrogen is transferred to methylene blue. The difference that may exist in this enzymatic action between the normal form and the G variant was determined by a study of the "resting" cells on a glucose substrate.

The method of obtaining resting bacteria was to streak a large number of Petri dishes containing nutrient agar and after the necessary incubation period (usually 24 to 48 hours) the growth on the plates was swept off with bent glass rods in sterile physiological salt solution. The combined suspensions from all the plates were filtered through sterile cotton in a funnel to eliminate pieces of agar and then centrifuged. The packed cells were washed twice with saline and the final packed cells, after drawing off the supernatant, constituted the "resting" cells used in these experiments.

In addition to the dysentery strains, both normal and G cultures of *Staphylococcus aureus* were included in some of the reduction tests. The Sonne G strains employed were G3, described previously as one that could be brought back to the normal colony size, and G7 which reverted to the normal several times but generally remained very minute under ordinary conditions. The *Staph. aureus* G strain designated as V8 was isolated by Swingle.¹⁰ This culture produced a very small colony on nutrient agar, about 0.15 mm. in diameter, but by certain methods of cultivation it could be brought back to the normal colony size. Upon reversion the usual biochemical and serological tests were produced indicating its relationship to the large colony form of *Staph. aureus*.

17. J. Hyg. **28**: 139, 1928.

18. J. Infect. Dis. **47**: 186, 1930.

19. Biochem. J. **20**: 166, 1926.

In the methylene blue reduction experiments the following reagents were used:

Buffer solution: 8.5 gm. NaCl, 0.2 gm. KH_2PO_4 , 0.12 gm. Na_2HPO_4 in 1,000 cc. distilled water.

Glucose: 2% solution.

Methylene blue: 1:5,000 solution.

Packed cells: These were generally used immediately after centrifugation. If action was too rapid the cells were diluted. It was found that if undiluted cells were used reduction took place too rapidly for accurate observation, often beginning before the tubes were placed in the water bath.

The apparatus consisted of a series of ten tubes each with a side arm connected to a main stem so that all the tubes could be evacuated at the same time. The number of cells used in each test was counted and weighed so that the rate of reduction per 100 million cells and per milligram of cells could be calculated.

The following table expresses the average results of eight tests with normal and G strains of *S. paradysenteriae* Sonne and four tests with normal and G strains of *Staph. aureus*. These tests were carried out under aerobic as well as anaerobic conditions.

TABLE 4.—*The Reduction of Methylene Blue by Normal and G Strains*

	<i>S. paradysenteriae</i> Sonne				<i>Staph. aureus</i>	
	269		Matheson		Normal	V8
	Normal	G3	Normal	G7		
Anaerobic						
0.1 cc. cells	3.4*	7.7	5.0	12.0	12.4	33.0
0.05 cc. cells	12.0	19.9	15.2	26.2		
Aerobic						
0.1 cc. cells	10.0	20.0	12.0	85.0		
0.05 cc. cells	35.0	45.0	30.0	322.0		
Time per 100 million cells						
Anaerobic	2.7	5.0	3.4	9.0	8.8	33.7
Aerobic	7.0	12.0	8.0	63.0		
Time per milligram cells						
Anaerobic	0.7	1.4	0.9	3.0	3.3	22.0
Aerobic	1.9	3.6	2.3	21.3		

* The reduction time expressed in minutes.

The results of these experiments show that the G variants bring about the reduction of methylene blue, in the presence of a suitable hydrogen donator such as glucose, at a slower rate than do the corresponding normal cells. The smaller the variant the greater was the time of reduction. G7, which consistently grew as a smaller colony, reduced methylene blue slower than G3 which in turn was slower than the normal strain. This was also seen in the staphylococcus strains. The rate of reduction appeared to be roughly proportional to the concentration of organisms present, a fact observed by Quastel.²⁰

Glucose is apparently a vigorous donator of hydrogen in the presence of normal *S. paradysenteriae* Sonne and normal *Staph. aureus* but its activity is greatly reduced in the presence of their corresponding small colony variants. It can therefore be said that the enzymatic action of the G variants

or the ability to transfer hydrogen from glucose to methylene blue is reduced. This diminished activity of the G colony form may explain the slow rate of fermentation in sugar broths.

CATAPHORETIC VELOCITIES OF NORMAL AND G FORMS OF *S. PARADYSENTERIAE*
SONNE AND OF *STAPH. AUREUS*

One property of small colony variants to which little or no attention has been paid is the electrophoretic mobility or cataphoretic velocity of the bacterial cell. This property of the bacterial cell, which is common to almost all minute or colloidal particles, has been associated and correlated with other properties in the study of bacterial forms. For instance agglutination or the stability of suspensions is correlated with virulence for certain animals. This stability has been found to be related to the electrophoretic potential of the cells.

Here a study has been made of normal and G forms of *S. paradysenteriae* Sonne and of *Staph. aureus* on the basis of their cataphoretic velocities.

The cultures used were the same as those employed in all previous experiments. The organisms were cultured on nutrient agar or veal infusion agar and after 24 hours the growth was suspended in sterile distilled water. This suspension was used for cataphoretic study. The p_H of the suspensions was from 6.8 to 7.0.

The apparatus used for the measurement of the velocities was the Northrop-Mudd assembly described by Jensen.²¹ The cell was carefully calibrated and the stationary layers determined. All measurements were made at one or the other of these layers. As a source of current "B" batteries were used (3 sections) giving an impressed voltage of 122 volts. The electrodes were made of zinc and immersed in zinc-sulfate solution. A reversing switch controlled the current so that the polarity of the electrodes could be quickly reversed.

Measurements were made by determining the time in seconds and tenths of a second that it takes one cell to traverse a distance of 100 microns as measured on the scale in the ocular micrometer. Ten such readings were made, five migrating in one direction and five in the reverse direction. The average of the ten readings was the velocity per 100 microns and from this the number of microns per second was calculated. From five to ten such determinations were made for each culture. Table 5 gives the microns-per-second average of each culture.

A direct relationship exists between cataphoretic velocity of the cells and size of the colony which the cells are capable of forming. The larger the colony the faster the velocity of the cells when corresponding normal and G strains are compared. For instance, the normal dysentery strain 269, whose colony diameter was 2.75 mm. had an average cell velocity of 19.5 microns per second while its corresponding G colony, with a diameter of 0.06 mm. had a cell velocity of 11.9 microns per second. Those G strains with intermediate colony sizes (partly reverted to the normal) as G3 (colony di-

21. *Oil and Soap* 9: 80, 1932.

ameter 0.50 mm.) had a somewhat higher velocity, 13.9 microns per second. This relationship of normal to variant was found to be true of all strains studied.

The cataphoretic velocity is related to the electrical charge upon the cell and a high velocity generally signifies a greater charge. The normal colony forms therefore possess a greater electrical charge than do the small colony variants.

TABLE 5.—*Cataphoretic Velocities of Normal and G Cultures*

Culture		Average Diameter of Colony in mm.	Average Velocity in μ -per-second*
<i>S. paradyssenteriae</i> Sonne			
Strain			
269	Normal	2.75	19.5
269	G2	0.06	11.9
269	G3	0.10	12.4
269	G3**	0.50	13.9
Matheson	Normal	2.50	16.8
Matheson	G7	0.05	9.4
Matheson	G7**	0.20	13.7
<i>Staph. aureus</i>			
Strain			
A	Normal	2.00	16.3
A	V8	0.25	9.5
A	V8**	1.00	12.8
M23	Normal	2.25	14.8
M23	V1	0.25	11.2
M23	V2	0.08	9.7
95	Normal	2.25	16.6
95	V4	0.15	11.3
95	V5	0.20	9.1
Norsenski	Normal	2.50	17.7
Norsenski	V6	0.16	9.0
Norsenski	V7**	1.25	12.6

* Average of 5 to 10 determinations.

** Small colony variants which have partially reverted to the normal.

FILTRATION EXPERIMENTS

The reports on the filterability of the small colony variants have shown no agreement. Kuhn and Sternberg²² found that their small colony forms of *E. coli*, *C. diphtheriae*, *E. typhosa* and *S. suipestifer* were filterable through Berkefeld N filters. Hadley, Delves and Klimek¹ demonstrated the filterability of the G forms of *S. dysenteriae* Shiga but Koser and Dienst³ found that the G variants of *S. paradyssenteriae* Sonne did not pass through Berkefeld filters. Hoffstadt and Youmans⁴ isolated filterable forms of *Staph. aureus* and Hadley and Carapetian⁵ obtained a G form of *E. typhosa* which was filterable through an N filter. Colien²³ obtained a filterable G variant of a yellow pigmented coccus. On the other hand Rettger and Gillespie⁶ were not able to demonstrate a filterable stage of *B. megatherium*. The G strains of *Staph. aureus* obtained by Swingle¹⁰ were not recovered in Berkefeld filtrates and Kopeloff⁹ found the small colony variants of *L. acidophilus* to be non-filterable.

The G strains used for the filtration experiments were those which constantly produced very minute colonies. They were G1, G6 and G7. Berkefeld

22. Zentral. f. Bakt. **121**: 113, 1931.

23. J. Bact. **30**: 301, 1935.

N and W filters and Seitz filters were completely assembled, sterilized at 15 pounds pressure for 20 minutes and tested by passing a 24 hour culture of *Chr. prodigiosum* through them. Those filters which prevented the passage of this organism were used. The cultures were inoculated into a number of tubes each containing 25 to 30 cc. of veal infusion broth and incubated at 37 C. Each strain was filtered after 2, 5, 8, 10 and 14 days through each filter. Records were kept of filters used, negative pressure and time. The amount of negative pressure applied was the lowest that would yield a filtrate within a reasonable time, usually equivalent to 2 to 3 inches of mercury. Fifteen to 20 cc. of culture at this pressure would take from 10 to 15 minutes to filter. After filtration four 0.1 cc. amounts of the filtrate were placed on marked spots on veal infusion agar plates and 1 cc. inoculated into veal infusion broth tubes. All transfers and the filtrate itself were incubated at 37 C. and observed daily. If the filtrate or tubes showed turbidity plates were streaked from them. If turbidity was not present plates were streaked after 2 and 4 days and the filtrates retained for 3 weeks for further observation.

A total of 45 filtrations was made, 15 with each strain. Strain G1 (269) was found to be filterable 8 times when passed through Berkefeld N filters but it did not pass through W or Seitz filters. Plates inoculated from the filtrate immediately after filtration were sterile but the tubes showed growth. The growth in the filtrates and tubes was streaked on veal infusion agar plates and typical G1 colonies were produced after 48 hours. The colonies of this strain were very small, 0.06 mm., and never reverted to the large colony form. The filtrates from strains G6 (St. Andrews) and G7 (Matheson) remained clear and plates and tubes inoculated from them showed no evidence of growth and were apparently not filterable. The fact that the filter-passing strain, G1, could not be made to revert to the normal form prohibits any definite conclusion concerning the filterability of the G derivatives of *S. paradysenteriae* Sonne.

DISCUSSION

Small colony variants of the G type occur irregularly and infrequently in cultures of *S. paradysenteriae* Sonne, and their presence depends upon the age of the culture. In some cases the variant may be of such a nature that reversion takes place more or less readily, while in others reversion is accomplished with difficulty or not at all. In the former case relationship to the large colony form can be demonstrated while in the latter the relationship cannot be definitely established and the validity of these variants remains doubtful.

From these studies no evidence could be found that the G variant is a stage in the so-called life cycle of the organism. If the organism passes through such a cycle under normal artificial cultivation the variants would probably occur with greater regularity and frequency. Unless methods are discovered to determine the exact factors which promote the G dissociation, the occurrence of the G variant can be said to be due to environmental factors involving a natural selection and the variants can be considered slow growing forms with or without the potentiality for reversion.

The variants are physiologically less active than the normal. Sugars are fermented very slowly due to the slow rate of cell multiplication and metabolic activity and in some cases the ability to attack carbohydrates is entirely lost. The antigenic structure and electrical charge of the variants are also modified and the extent of this change seems to depend upon the degree of dissociation.

The fact that different degrees of dissociation can occur may explain the divergent results in regard to antigenicity, biochemical activity and filterability obtained by those who have studied the G variant.

SUMMARY

The G colony variant of *Shigella paradysenteriae* Sonne occurs infrequently and irregularly in aging agar slant cultures. Several strains were isolated by streaking nutrient agar plates from old cultures.

The G colonies were generally very minute and some reverted to the large colony form with difficulty. Some strains could not be brought back to the normal colony size. The cell morphology was irregular and rod, coccus and filamentous forms were present. Cell division of the variant differed somewhat from the normal and reproduction was slow.

The biochemical activity of the minute colony forms was greatly reduced. Sugars were fermented very slowly and in some cases the ability to attack carbohydrates was lost. Methylene blue was reduced more slowly by the variant and the rate varied with the size of the colony.

Serological tests showed some of the variants to be related to the normal. The G strains which reverted were agglutinated by normal antiserum to a high titer.

By cataphoretic studies it was shown that the speed of migration of the variant was slower than the normal. Intermediate colony-size variants had velocity rates ranging between those of the normal and the variant. This indicated that the charge on the variant was lower.

The variants, with one exception, were not filterable through Berkefeld N and W candles or Seitz filters. One G colony form, which did not revert to the normal, passed through N filters about 50% of the time.

